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Up-regulation of cytokine mRNA in human monocytes and myeloid cell lines by the differentiation/activation factor p48

D. P. KESTLER, S. AGARWAL & R. E. HALL *Department of Medicine, Division of Hematology/Oncology, University of Tennessee Medical Center/Graduate School of Medicine, Knoxville, Tennessee, USA*

SUMMARY

Polypeptide 48 is a 48 000 MW protein, originally isolated from conditioned media of some human leukaemic cell lines, that induces differentiation and cytolytic activity in HL-60 promyelocytic leukaemia cells and activates human peripheral blood monocytes to secrete interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α). In the present study we examined the effects of p48 on the accumulation of a series of monokine transcripts, including TNF-α, IL-1α, IL-1β and IL-6, in human peripheral blood monocytes and the myeloid/monocyte cell lines HL-60 and U937. Using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot analysis, p48 was found to induce accumulation of TNF-α, IL-1α and IL-1β mRNA in peripheral blood monocytes, HL-60 and U937 cells. IL-6 mRNA was found to be increased in p48-stimulated peripheral blood monocytes but not HL-60 or U937. Thus, the secretion of IL-1 and TNF-α by p48-stimulated monocytic cells was associated with up-regulation of cytokine mRNA, suggesting that p48 leads to increased transcription or mRNA stability in these cells. As U937 and HL-60 are likly to represent premonocyte stages of haemopoietic differentiation, it is possible that the effect of p48 on IL-6 mRNA, in contrast to its effect on TNF and IL-1, requires cells to be at a later differentiation step.

INTRODUCTION

The process of haemopoietic cell differentiation is not completely understood, but several factors have been identified that facilitate differentiation of haemopoietic and lymphoid cells. A number of cytokines is known to stimulate differentiation *in vitro*, including the colony stimulating factors (CSF), tumour necrosis factor-α (TNF-α), TNF-β, interferon-γ (IFN-γ), IL-6, transforming growth factor-β (TGF-β) and leukaemia inhibitory factor.[1–7] Some of these cytokines are known to differentiate cells and also to modulate cellular functions and to induce other cytokines.[8–11] For example, IFN-γ has been shown to activate cytotoxic function in a variety of cells,[12] as well as up-regulate other cytokines including IL-1, IL-6 and TNF.[13] In addition, IL-1 and TNF-α are known to up-regulate each other as well as themselves[14,15] One current model of cytokine action is that cytokines may not act alone but may act either in concert with other cytokines, or alternatively in a cascading fashion with sequential induction of subsets of cytokines.[16]

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Abbreviations: CSF, colony-stimulating factor; FCS, fetal calf serum; LPS, lipopolysaccharide; PCR, polymerase chain reaction; RT, reverse transcriptase; SSC, standard saline citrate; TNF, tumour necrosis factor.

Correspondence: Dr D. P. Kestler, Memorial Research Center, Room 216, University of Tennessee Medical Center, Knoxville, TN 37920, USA.

Our laboratory has previously identified, purified from conditioned media of human leukaemic cell lines, and extensively characterized a 48 000 MW polypeptide (termed p48) with haemopoietic differentiation activities.[17,18] Polypeptide 48 induces monocytic differentiation and cytolytic activity in HL-60 (promyelocytic leukaemia) cells and is antiproliferative (but not non-specifically toxic or lytic) to several other tumour cell lines. In addition, p48 has been found to induce secretion of IL-1 and TNF-α by human peripheral blood monocytes.[19] Like a number of other cytokines, p48 has also been shown to exist in a biologically active, integral membrane form.[20] Polypeptide 48 has been shown to be immunologically and biologically distinct from TNF-α and TNF-β, IFN-α and IFN-γ, macrophage (M)-CSF, IL-6, and other biological molecules with differentiation activity.[17,18] The N-terminus of the protein has been sequenced and the gene encoding for this protein has recently been cloned and found to be novel (R. E. Hall, S. Agarwal & D. P. Kestler, submitted for publication).

In order to characterize further the immunomodulatory effects of p48, we have examined the possibility that p48 regulates cytokine mRNA expression in monocytes and the myeloid/monocytic cell lines HL-60 and U937. Here we report the effect of p48 on mRNA transcript levels of IL-1α and IL-β, TNF-α and IL-6.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS; phenol extract from *Salmonella*

typhosa) was obtained from Sigma Chemical Co. (St Louis, MO), dissolved in RPMI-1640 medium at 2 mg/ml and stored at −20° prior to use. Reagents for cDNA synthesis were obtained from Bethesda Research Laboratories (BRL, Bethesda, MD) and Promega Corporation (Madison, WI). Polymerase chain reaction (PCR) reagents were obtained from Pharmacia (Piscataway, NJ) and BRL. PCR synthesis was perfomed using a Gene Amp 9600 system (Perkin Elmer-Cetus, Norwalk, CT). Oligonucleotide primers were synthesized on an Applied Biosystems Model 391 automated DNA synthesizer (Foster City, CA). The oligonucleotide sequences employed in this study were previously used in reverse transcriptase (RT)-PCR analysis of TNF-α, IL-1α and IL-1β, IL-6 and β-actin.[21]

Cell lines

HL-60 (human promyelocytic leukaemia) and U937 (human histiocytic lymphoma) cell lines were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin (complete medium). Reh (human pre-B leukaemia) was adapted to grow in medium containing 2% FCS, as described previously.[18]

Polypeptide 48 differentiation/activation factor and rabbit anti-p48 antiserum

Polypeptide 48 was partially purified by 20% ammonium sulphate precipitation from conditioned medium of the Reh cell line adapted to grow in 2% FCS medium. Purified p48 was prepared by preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and monospecific neutralizing antiserum was prepared in rabbits as described elsewhere.[18]

Purification and stimulation of monocytes and cell lines

Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque centrifugation of heparinized venous blood from healthy volunteers, as described elsewhere.[19] Cells (10^7/ml/well) were plated into 24-well (16-mm diameter) tissue culture plates (Costar, Cambridge, MA), allowed to adhere for 2 hr (5% CO_2/95% air) at 37°, and non-adherent cells removed by vigorous washing. Adherent cells (>90% monocytes by Giemsa staining) or washed cell line cells were cultured for various times with LPS, partially purified p48 or purified p48 in complete medium at 37°. After stimulation the monocytes were removed by incubation (5°, 30 min) with 1 ml/well of 1 mM EDTA in phosphate-buffered saline (PBS), followed by repeated pipetting. Stimulated monocytes and cell lines were centrifuged at 250 ***g*** for 7·5 min, and then RNA extracted immediately.

RNA isolation and cDNA synthesis

Total cellular RNA was isolated by the guanidinium isothiocyanate/acid phenol–chloroform method,[22] dissolved in 100 μl of diethylpyrocarbonate-treated water (DEPC-water), adjusted to 250 mM sodium acetate (pH 7·0) and precipitated with 2·5 volumes of absolute ethanol at −80° for 1 hr. Precipitates were washed three times with cold 75% ethanol, dried under vacuum, and dissolved in 10 μl of 1 mM dithiothreitol containing 1 U/ul RNasin ribonuclease inhibitor (Promega). Prior to cDNA synthesis, the RNA was incubated at 60° for 2 min and then chilled on ice. RNA equivalent to 200 000 cells was used to make cDNA in a 15-μl RT reaction containing 500 μM deoxynucleotide triphosphate, 50 mM Tris–HCl (pH 8·2), 12 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0·5 mM spermidine, 50 μg/ml oligo-dT (12–18 mer) and 4 U AMV RT (Promega). The reactions were incubated at 42° for 90 min.

PCR amplification

A 5-μl aliquot of the cDNA product was added to a PCR mix containing approximately 1 μM each of the appropriate forward and reverse primers, 1·25 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl (pH 8·4), 20 μg/ml gelatin, 200 μM deoxynucleotide triphosphate and 0·4 U Taq DNA polymerase in a final volume of 20 μl. The amplification reaction consisted of 25 cycles of 94° denaturation (1 min), 55° annealing (1 min) and 72° synthesis (1 min), with a final extension at 72° for 15 min. The final PCR products were analysed by electrophoresis (50 V, 15 mA) on 1·5% agarose gels in 40 mM Tris–acetate (pH 8·5) and 2 mM EDTA. *Hae*III-digested *Phi*X174 replicative-form (RF) DNA fragments were used as molecular weight standards. Some gels were subsequently transferred to nylon membranes for Southern analysis and probed with cytokine-specific cDNA fragments isolated from agarose gels that had been nick-translated with ^{32}P-dATP and ^{32}P-dCTP (3000 Ci/mM) and purified over G-50 Sephadex spin columns.[23] Hybridization (65°, 16 hr) was in the presence of 6× SSC, 5× Denhardt's solution, 0·5% SDS, 150 μg/ml herring sperm DNA and 1×10^6 c.p.m./ml heat-denatured cDNA probe. The blots were washed to a stringency of 0·2× SSC at 65° and then developed against Kodak XAR film with Dupont Lightning Plus (Wilmington, DE) screens at −80°.

Northern blots

Procedures were performed according to established methods.[23] Briefly, RNA samples, 7·5 μg/15 μl in 20 mM 3′-(*N*-morpholino)-propanesulphonic acid (MOPS; pH 8·0), 1 mM EDTA, 50% formamide, 6·5% formaldehyde and 0·4% xylene cyanole/bromophenol blue, were heated at 55° for 15 min and then cooled on ice. The RNA samples were then analysed by electrophoresis on 1·2% agarose gels in 20 mM MOPS (pH 8·0)–1 mM EDTA–1·1% formaldehyde. Lanes containing a RNA ladder (BRL) and ribosomal RNA for size reference were stained with ethidium bromide (0·5 μg/ml) and photographed under UV transillumination. Gel portions to be transferred were washed twice (30 min each) in DEPC–water, once in 10× SSC, and then transferred (16 hr, room temperature) by imbibition onto Biotrans nylon membranes (ICN, Irvine, CA) employing a wick and 10× SSC. The blots were rinsed in 4× SSC, baked at 80° for 90 min under vacuum, and incubated (41°, 6 hr) in prehybridization buffer consisting of 10× SSC, 5× Denhardt's solution, 50% formamide, 0·5% SDS, 0·1% sodium pyrophosphate and 150 μg/ml denatured herring sperm DNA. Hybridization was carried out at 41° for 16 hr with a ^{32}P heat-denatured DNA probe (10^6 c.p.m./ml, specific activity $>10^8$ c.p.m./μg DNA) in prehybridization buffer containing 10% dextran sulphate, followed by washing twice (15 min, room temperature) in 3× SSC and then at 55° to a stringency of 0·1× SSC. The blots were then exposed to film and developed as described above. cDNA probes used were as follows: TNF-α, 820 bp *Eco*R1 fragment of λ42-4 (a generous gift from Dr A. Singh, Genentech Inc., South San Francisco, CA); IL-1α and IL-1β, clones phIL1AcDNA and YEpsec1-hIlbeta from American Type Culture Collection (ATCC, Rockville, MD), and β-actin, a 548 bp RT-PCR fragment

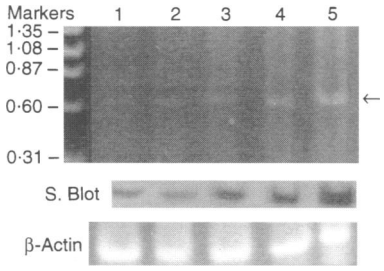

Figure 1. TNF-α RT-PCR product from monocytes cultured for 5 hr with partially purified p48 or LPS. The upper panel is an ethidium bromide-stained agarose gel with lanes: (1) no additions; (2) 2·5 μg/ml p48 and 1/50 anti-p48 rabbit sera; (3) 1 μg/ml LPS; (4) 2·5 μg/ml p48; (5) 2·5 μg/ml p48 and 1/50 normal rabbit sera. The arrow indicates the position of the 702 bp TNF-α PCR-generated fragment. Markers are *Hae*III-digested *Phi*X174 RF DNA with fragment sizes shown in kilobase pairs (kbp). The middle panel shows an autoradiogram from Southern analysis of the TNF-α PCR products probed with ^{32}P-labelled TNF-α cDNA. The lower panel displays the ethidium bromide gel profile of β-actin RT-PCR products generated from sample RNA employed in the upper and middle panels.

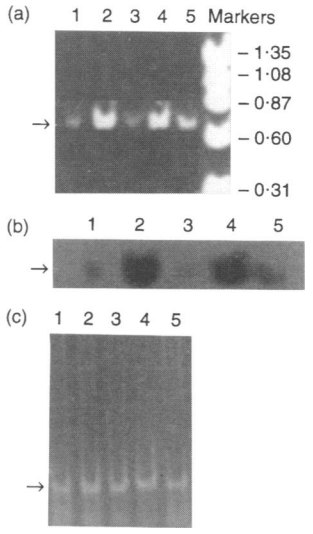

Figure 2. TNF-α RT-PCR product from HL-60 cells cultured for 5 hr with partially purified p48 or LPS. (a) Lanes: (1) no additions; (2) LPS 1 μg/ml; (3) p48 2·5 μg/ml and 1/50 rabbit anti-p48; (4) 2·5 μg/ml p48; (5) 2·5 μg/ml p48 and 1/50 normal rabbit serum. The arrow indicates the position of the 702 bp TNF-α PCR product. Markers are the same as in Fig. 1. (b) Autoradiogram of (a) blotted and probed with a ^{32}P-TNF-α cDNA probe. (c) Ethidium bromide-stained gel of β-actin-primed RT-PCR product of sample RNAs employed for (a) and (b). The arrow indicates the position of the 548 bp β-actin PCR product.

beginning 102 nucleotides downstream from the ATG start codon.[21]

RESULTS

Our laboratory has reported previously that p48 stimulates monocytes to secrete TNF-α and IL-1 proteins,[19] and we have begun to examine the mechanism of this induction. Figure 1 presents the effects of partially purified p48 on levels of TNF-α mRNA in peripheral blood monocytes after 5 hr cell culture. TNF-α mRNA was assayed using RT-PCR methods with TNF-α-specific primers as published previously.[21] In preliminary experiments we found a roughly linear dose–response relationship for TNF-α mRNA up to at least 2·5 μg/ml partially purified p48, and we found no appreciable difference in mRNA accumulation in monocytes cultured for 5 or 15 hr (data not shown). In addition, there was a roughly linear dose–response relationship for RT-PCR up to at least 25 cycles, as has been observed for cytokines by other investigators.[24,25] In this and other experiments employing anti-p48 antibodies added to cultures, we chose the shorter time-point in order to minimize possible metabolic effects of the monocytes on antigen–antibody complexes. PCR products were detected by ethidium bromide staining of agarose gels and/or Southern blotting followed by hybridization with ^{32}P-labelled cDNA probes.[26] Unstimulated monocytes contained a low level of TNF-α mRNA (Fig. 1, upper panel, lane 1), whereas both LPS and p48 up-regulated TNF-α mRNA (lanes 3 and 4). Stimulation of TNF-α mRNA by p48 was inhibited with anti-p48 rabbit antiserum, in contrast to normal rabbit serum control which had a slightly stimulatory effect (lanes 2 and 5). This inhibition by anti-p48, as well as previously reported experiments showing that p48-induced TNF was not inhibited by polymyxin B,[19] represent strong evidence that the p48 effect is not due to contaminating endotoxin. The level and integrity of monocyte RNA appeared similar in all lanes, as judged by the level of β-actin RT-PCR product (Fig. 1, lower panel).

Figure 2 shows a similar analysis of the effects of p48 on TNF-α mRNA in HL-60 cells, a human promyelocytic cell line with monocyte features. Some endogenous TNF-α mRNA was detected in unstimulated cells after 5 hr culture (Fig. 2a, lane 1), but this was markedly increased in response to p48 (lane 4). LPS also enhanced TNF-α mRNA accumulation in HL-60 cells (lane 2). Stimulation of TNF-α mRNA by p48 was markedly inhibited by rabbit anti-p48 antiserum (lane 3). Normal rabbit serum at the same dilution also slightly inhibited p48-stimulated TNF-α mRNA in HL-60 cells (lane 5), but inhibition was markedly less than that of rabbit anti-p48 antiserum. The reason for this non-specific inhibition by normal rabbit serum is not known. Hybridization analysis of these products with a ^{32}P-labelled TNF-α cDNA probe confirmed the results observed by direct visualization of ethidium-stained gels (Fig. 2b). Similar RNA levels in the samples were again demonstrated in the actin controls (Fig. 2c).

Stimulation of TNF-α mRNA by p48 was also observed in U937 cells after 15 hr incubation, whereas minimal stimulation was observed at 5 hr (data not shown). U937 (histiocytic lymphoma) cells are felt to represent a more mature stage of monocyte differentiation than HL-60 cells, but a less committed state than peripheral blood monocytes.[27]

The effects of p48 on IL-1 mRNA accumulation was also examined. Our laboratory has reported previously that p48 stimulates secretion of IL-1 activity by human peripheral blood monocytes, but it was not determined whether this represented IL-1α, IL-1β or both.[19] As shown in Fig. 3a, B, p48 up-regulated IL-1α mRNA in both HL-60 cells and human peripheral blood monocytes. Similar to TNF-α, stimulation of IL-1α mRNA by p48 was dose-dependent over the range of 0–2 μg/ml. In contrast to TNF-α, stimulation of IL-1α mRNA

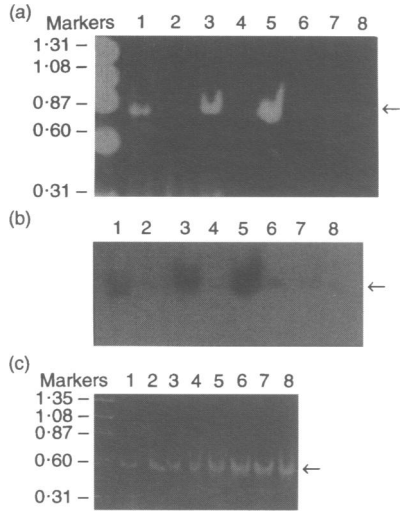

Figure 3. IL-1α RT-PCR product from HL-60 cells and peripheral blood monocytes cultured for 15 hr with partially purified p48 or LPS. (a) Ethidium bromide-stained gel with lanes: (1) HL-60, 2 μg/ml p48; (2) HL60, 0·5 μg/ml p48; (3) HL-60, 1 μg/ml LPS; (4) HL-60, no additions; (5) monocytes, 2 μg/ml p48; (6) monocytes, 0·5 μg/ml p48; (7) monocytes, 1 μg/ml LPS; (8) monocytes, no additions. The arrow indicates the position of the 810 bp IL-1α RT-PCR product. Markers are detailed in the legend to Fig. 1. (b) Southern blot analysis of IL-1α RT-PCR products of (a) using a ^{32}P-labelled IL-1α cDNA probe. The arrow indicates the position of the IL-1α hybridization signal. (c) Ethidium bromide-stained gel analysis of β-actin RT-PCR product from the same RNA samples as in (a). The arrow indicates the position of the 548 bp β-actin product.

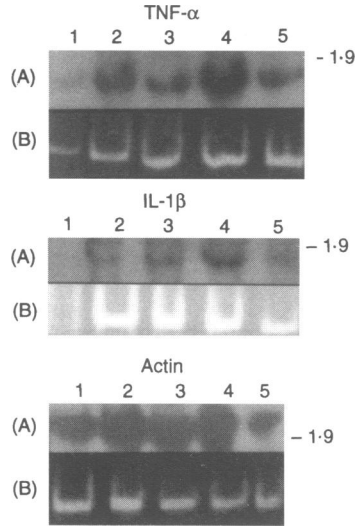

Figure 5. Northern blot and RT-PCR analysis of HL-60 cells stimulated with purified p48. HL-60 cells were cultured for 15 hr in the presence and absence of stimulants followed by extraction of RNA and (A) Northern blot analysis or (B) RT-PCR analysis. RNA was analysed by (A) hybridization with ^{32}P-labelled cDNA probes or (B) RT/PCR using specific PCR primers for TNF-α (upper panels), IL-1β (middle panels) or β-actin (lower panels). Lanes are as follows: (1) no addition; (2) LPS 1 μg/ml; (3) purified p48 100 ng/ml; (4) purified p48 300 ng/ml; (5) partially purified p48 (20% ammonium sulphate precipitate of Reh-conditioned medium) 2·5 μg/ml. The marker at the left of (A) is in kb and represents the closest RNA marker to the hybridization signal.

was only minimal at 5 hr (data not shown) but appreciable at 15 hr of culture. Actin controls revealed similar integrity and amount of sample loaded (Fig. 3c).

Similarly, p48 was found to increase IL-1β mRNA levels in both peripheral blood monocytes and HL-60 cells after 15 hr

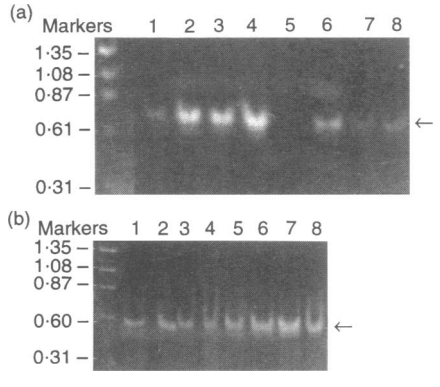

Figure 4. IL-1β RT-PCR product from HL-60 cells and peripheral blood monocytes cultured for 15 hr with partially purified p48 or LPS. (a) Ethidium bromide-stained gel with lanes: (1) monocytes, no additions; (2) monocytes, 1 μg/ml LPS; (3) monocytes, 0·5 μg/ml p48; (4) monocytes, 2 μg/ml p48; (5) HL-60, no additions; (6) HL-60, 1 μg/ml LPS; (7) HL-60, 0·5 μg/ml p48; (8) HL-60, 2 μg/ml p48. The arrow indicates the position of the 820 bp IL-1β RT-PCR product. Markers are detailed in the legend to Fig. 1. (b) β-actin RT-PCR product from the same RNA samples as in (a). The loading order is the reverse of (a). The arrow indicates the position of the 548 bp β-actin product.

culture (Fig. 4), but was more stimulatory in monocytes. Similar to the findings regarding TNF-α mRNA, p48-induced up-regulation of IL-1β was markedly inhibited by anti-p48 antiserum (data not shown).

In order to confirm these findings further using RT-PCR, we performed Northern blot experiments using HL-60 cells stimulated with LPS or purified p48.[18] Probing with ^{32}P-labelled TNF-α, IL-1β, (or IL-1α, data not shown) cDNA confirmed the stimulation of these transcripts observed using RT-PCR (Fig. 5).

Finally, we examined the effect of p48 on the modulation of IL-6 mRNA (Fig. 6). We found no RT-PCR-detectable IL-6 mRNA in HL-60 or U937 cells, either unstimulated or stimulated with p48 or LPS (data not shown). In contrast to these monocyte-like cell lines, p48 up-regulated IL-6 mRNA levels in human peripheral blood monocytes at both 5 and 15 hr of culture. LPS-stimulated monocytes also contained detectable IL-6 mRNA, as has been reported previously by others.[28] In addition to the predicted 0·64 kb IL-6 RT-PCR product, a second product was observed consistently at 0·45 kb, which we have shown to be an alternatively spliced form of IL-6.[29,30]

DISCUSSION

TNF-α, IL-1, and IL-6 are three immunomodulatory/haemopoietic cytokines with overlapping biological activities, including inflammatory, tumoricidal and haemopoietic activities.[31–33] Our laboratory has previously identified, purified and extensively characterized a 48 000 MW protein (termed p48) that possesses a number of monocytic differentiation and activation

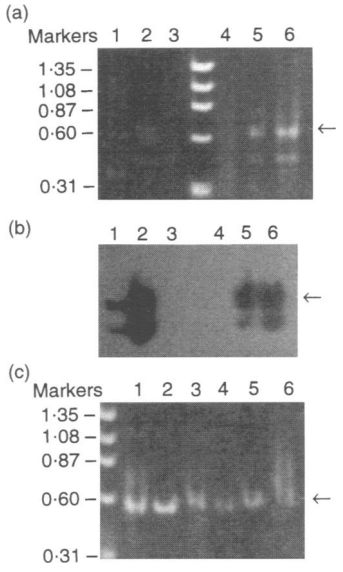

Figure 6. IL-6 RT-PCR products from peripheral blood monocytes cultured for 15 hr with partially purified p48 or LPS. (a) Ethidium bromide-stained gel of IL-6 RT-PCR products from monocyte cultures from two different donors. Lanes 1–3 (donor 1) monocyte culture additions were: (1) p48 2 μg/ml; (2) LPS 1 μg/ml; (3) no additions. Lanes 4–6 (donor 2) monocyte culture additions were: (4) no additions; (5) LPS 1 μg/ml; (6) p48 2 μg/ml. The arrow indicates the position of the 636 bp IL-6 major predicted RT-PCR product. Markers are the same as in Fig. 1. In addition to the predicted 636 bp IL-6 RT-PCR product, a second product was consistently observed at 0·45 kb, which we have shown to be an alternatively spliced form of IL-6.[29,30] (b) Southern blot analysis of (a) hybridized with a ^{32}P-IL-6 cDNA probe. The arrow indicates the position of the IL-6 hybridization signal. Note that the ethidium-stained IL-6 signal is diminished for donor 1 (a) but is easily detectable on Southern blotting. (c) β-actin RT-PCR product profile of the same sample RNA used in (a) and (b). The arrow indicates the 548 bp β-actin RT-PCR product.

properties.[17–19] This protein was originally isolated from the conditioned media of some human leukaemia cell lines, and later shown to exist also as a membrane-associated form.[20] We have reported previously that p48 induces fresh human peripheral blood monocytes to secrete TNF-α and IL-1.[19] In the current study we have shown that p48 induces accumulation of mRNA for IL-1α and IL-1β, and TNF-α in human monocytes and HL-60 and U937 cell lines, and for IL-6 in monocytes (but not HL-60 and U937). Other laboratories have previously reported up-regulation of TNF-α and IL-1 mRNA by myeloid differentiation agents such as phorbol myristate acetate, dimethyl sulphoxide and retinoic acid.[34,35] Since HL-60 and U937 are likely to represent premonocytic stages of differentiation, these data suggest that the p48 effect on IL-6 mRNA, in contrast to the effect on IL-1 and TNF-α mRNA, requires cells to be at a later differentiation step. In support of this, HL-60 and U937 cells have been reported to make IL-6 mRNA if treated with phorbol myristate acetate.[36]

In addition to the overlapping biological activities of IL-1, TNF-α and IL-6, each of these cytokines is known to modulate expression of themselves and others within this group and are co-induced or inhibited by other non-cytokine stimulators. Current evidence, primarily using recombinant cytokines *in vitro*, suggests that these and other cytokines act in a network or cascading fashion, and that different combinations of cytokines may produce different biological activities.[16] Exactly how different cytokines relate to each other as modulators of immune effector cell activities and as haemopoietic growth and differentiation factors is under intense investigation in many laboratories.

Our laboratory has recently isolated a cDNA clone encoding for a protein that reacts with anti-p48 antibodies, and thus is the cDNA for the putative p48 gene (R. E. Hall, S. Agarwal, D. P. Kestler & E. J. A. Cobb, submitted for publication). This cDNA clone has been sequenced and represents a novel gene. Further characterization of the effects of p48 on cytokine gene expression, as well as the receptor/signal transduction mechanism for p48, should lead to important new information regarding control of expression and modulation of TNF-α, IL-1, IL-6 and possibly other inflammatory and haemopoietic cytokines.

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